THE VIRULENCE OF OLD AND OF RECENT CULTURES OF *BACILLUS PESTIS.**

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THE virulence of *B. pestis* when grown for a long time upon artificial media is a subject about which the evidence is conflicting. Some writers state that the virulence of the organism is soon lost; others hold that it is maintained for a long period.

I have had the opportunity to determine the virulence of a number of old strains of *B. pestis* and to compare it with the virulence of cultures of the organism that have been isolated during the recent plague campaign in San Francisco.

Source of cultures.—In this work I have used the eight stock cultures of *B. pestis* at the Hygienic Laboratory of the Public Health and Marine Hospital Service, Washington, D. C.[1] Unfortunately no accurate history of the majority of these cultures was to be obtained. They had, with one exception, been at the Hygienic Laboratory for several years; some of them for many years. The culture designated "X" had been in stock only a few months. They were kept on agar in sealed tubes in a dark room at a constant temperature of about 16° C. Every three or four months the cultures were transplanted to a new tube of agar, grown for a few days in the incubator at 37° C., and after a good growth was obtained they were returned to the cold room. It could not be learned when the cultures were passed through animals; that is, when they were inoculated into animals, recovered by culture methods from the body of the animal after death, and then returned to the stock. **It is definitely known that this had not been done for at least eight months before I began this work,** and it is very improbable that they had been treated in this manner for at least two years preceding these experiments. Such histories as were obtainable of the old cultures that proved virulent are given in connection with the tables showing the influence of graduated doses of

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[1] I wish to acknowledge my indebtedness to Surgeon M. J. Rosenau, U. S. P. H. and M. H. S., Director of the Hygienic Laboratory, for the privilege of using these cultures.

the organism. The cultures are called by the names of the places where they were isolated, Manila, Bombay, Jedda, New York, Glasgow, X (unknown), Frisco (San Francisco), Reedy Island. The cultures Bombay and Frisco proved to be practically avirulent. It was not known exactly how long these cultures had been at the laboratory, but Culture Frisco was isolated during the first plague campaign in San Francisco (1900–1904).

We have complete and accurate information concerning the strains of the organisms isolated in San Francisco during the recent plague campaign. These cultures are named to correspond with the animal from which they were isolated and with a number in our laboratory records; Human No. 171, Rat No. 66, Rat No. 82, Squirrel No. 1. Their histories are given in connection with the same series of tables as the old cultures.

Verification of cultures.—All of the old cultures, as well as the ones isolated in San Francisco, were carefully tested culturally and found to agree with *B. pestis* in every particular. The lesions produced in rats and in guinea-pigs by Manila, New York, Glasgow, X, Jedda, Rat No. 66, Rat No. 82, Human No. 171, and Squirrel No. 1 were characteristic of plague in these animals. Reedy Island produced typical lesions of plague in guinea-pigs. Frisco and Bombay killed guinea-pigs only when the animals were given the culture intraperitoneally, and the lesions were generally not those of acute plague. These two strains sometimes gave rise to the lesions of subacute or chronic plague. The white rats inoculated with Reedy Island, Frisco, and Bombay did not die so that in the case of these three cultures we lacked the characteristic lesions in these animals as comfirmatory evidence of the nature of the organisms. There is no reason, however, for doubting that these avirulent cultures are strains of the true *B. pestis*.

The question presented itself as to whether these cultures of low virulence might be examples of *B. pseudotuberculosis rodentium* (Pfeiffer), but after comparing them with several strains of the latter organism I am convinced that such is not the case. The culture Reedy Island is known to have been isolated from a case of human plague and it is fair to assume that the same is true of the cultures Frisco and Bombay. The eight old strains were tested culturally by Acting Assistant Surgeon W. B. Wherry, who confirmed my findings. Dr.

Wherry investigated the reactions of these cultures on a series of sugar broths and they were found to give the fermentative reactions of *B. pestis*. He has kindly furnished me with the results of his work with the carbohydrates. "The cultures were grown at 36°–37° C. in +1 broth containing litmus and 1 per cent of various carbohydrates. They all produced acid (but no gas) from dextrose, levulose, galactose, maltose, and mannite, but did not ferment lactose, saccharose, nor inulin. The fermentative activity of the cultures was alike throughout—dextrose, levulose, and galactose being most actively fermented, mannite next, and maltose least. Throughout the series maltose was broken down only during the second 24 hours."

EXPLANATION OF TERMS USED IN TABLES.

Day of death.—The animals generally died a fraction of a day earlier than is shown in the tables, as all that died after 4 P. M. on any day were counted as dying on the succeeding day.

Lesions.—The lesions in guinea-pigs were regarded as those of **acute plague** when there was a brawny, bloody, or gelatinous local reaction, one or more caseous buboes surrounded by exudate which was usually bloody, an enlarged, friable spleen with many whitish granules; of **subacute plague,** when there was one or more caseous or purulent buboes with dense, tough capsules; with or without caseous or purulent foci in the spleen or in the lungs. The cases called subacute, or some of them, might with equal propriety be called chronic plague. The lesions of acute plague in the rat are a general subcutaneous injection, a bubo, a granular liver, an enlarged, firm spleen, and a pleural effusion. Smears were usually made for the purpose of demonstrating pest-like organisms. No record is made here of the results of the examination of smear preparations.

B. pestis recovered.—This refers to the recovery and identification of the organism by culture methods. Cultures were made from only one organ in the case of each animal and for convenience we generally made stroke cultures from the liver. The organism could have been recovered more frequently by the use of the plate method and by making cultures from more than one tissue, but on account of the limited time at my disposal this was not practicable. The identification of the organisms recovered was based on the character and appearance of the colonies on agar, the nature of the growth in broth, and the production of characteristic involution forms on 3 per cent salt agar. These three points are ample for establishing the identity of an organism as *B. pestis* when it is isolated from an animal having the typical gross lesions of plague.

The statement as to the generation of the organism used in the case of cultures Manila, New York, Glasgow, Bombay, Frisco, Jedda, X, and Reedy Island has reference to the generation on artificial media (agar) **after the cultures came into my possession at which time they had been grown for at least eight months on agar.** In the case of the cultures Human No. 171, Rat No. 66, Rat No. 82, and Squirrel No. 1 it refers to the generation after the original isolation from the naturally infected animal from which the culture was obtained.

The platinum loop used throughout the series of experiments was ellipsoid in shape, 1 mm. in its long diameter by 0.5 mm. in the short one. A calculation based

on the dimensions of this loop and upon the size of *B. pestis* indicated that it would take up about 260,000,000 of the organisms from an agar growth.

VIRULENCE FOR GUINEA-PIGS

Preliminary experiment.—The first experiment was made by inoculating guinea-pigs by Kölles (cutaneous) method with a three-day-old agar culture, second generation. The results are shown in Table 1. One loopful of culture was used in each case.

TABLE 1.

Culture	Weight of Guinea-Pig	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
Jedda	235 gm.	7	Acute plague	Yes	
Manila	260 gm.	6	" "	"	
New York		8	" "	Contaminated	
Glasgow	250 gm.	7	" "	Yes	
X		8	" "	"	
Reedy Island	245 gm.	7	" "	No growth	
Bombay			None	" "	Killed 21st day
Frisco			"	" "	" " "

Influence of size of dose.—The results obtained with old cultures that were supposed to require large doses subcutaneously to bring about a fatal infection were so surprising that it seemed desirable to investigate the subject further.

In each case a 24-hour agar culture was used; second generation. One guinea-pig was inoculated by the cutaneous method with one loopful of a suspension of the culture in physiological salt solution. The suspension was made to approximate the turbidity of a 24-hour broth typhoid culture. The other animal was given subcutaneously an entire agar culture suspended in salt solution. These quantities are obviously very inexact, but the latter dose was many hundred times the size of the former. In the table the first animal (A) under the head of each culture received the smaller dose; the second (B) received the larger dose.

TABLE 2.

Culture		Weight of Guinea-Pig	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
Jedda	A	658 gm.	5	Acute plague	Yes	
	B	510 gm.	3	" "	"	
Manila	A	495 gm.	8	" "	"	
	B	490 gm.	7	" "	"	
New York	A	345 gm.	7	" "	"	
	B	337 gm.	5	" "	"	
Glasgow	A	400 gm.	6	" "	"	
	B	400 gm.	5	" "	"	
X	A	430 gm.	11	Subacute "	"	
	B	407 gm.	4	Acute "	"	
Reedy Island	A	470 gm.		Subacute "	No	Killed 25th day
	B	360 gm.		Enl'g'd Glands	"	" " "
Bombay	A	374 gm.		None	"	" 17th "
	B	440 gm.		Abscess at site	"	" 14th "
Frisco	A	420 gm.		None	"	" 14th "
	B	433 gm.		Abscess at site	"	" 14th "

The animals receiving the larger dose died earlier than the others, but except in the case of Culture X, the difference was not striking.

Passage through a guinea-pig.—The next series was undertaken for the purpose of ascertaining whether there was any change in the virulence of the organism due to its passage through a guinea-pig.

The first animal (A) under each culture was inoculated by the cutaneous method directly from the spleen of the animal in the preceding experiment that had been given an agar slant of culture subcutaneously. The control (B) was inoculated in the same manner (cutaneously) but with one loopful of a suspension of a 24-hour agar culture of approximately the turbidity of a 24-hour typhoid culture in broth. The culture was the second generation and was 24 hours old.

As the animals inoculated with Reedy Island, Bombay, and Frisco in the preceding experiment did not die they could not be made a part of the present experiment.

TABLE 3.

Culture		Weight of Guinea-Pig	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
edda.....	A	345 gm.	7	Acute plague	Contaminated	
	B	371 gm.	5	" "	Yes	Control
Manila.....	A	389 gm.	not recorded	" "	"	
	B	384 gm.	16	Subacute "	No growth	Control—Death probably due to pneumonia
New York.....	A	322 gm.	9	Acute plague	Yes	
	B*	345 gm.	7	" "	"	Control
Glasgow.....	A	365 gm.	7	" "	"	
	B	400 gm.	8		"	"
X.....	A	311 gm.	5	" "	"	
	B*	430 gm.	11	Subacute "	"	"

* Data as to these guinea-pigs carried from preceding table to provide controls.

It will be observed that in the case of the cultures Jedda and New York the animals inoculated from the culture died earlier than did those inoculated from the spleen; on the other hand in the case of the cultures Glasgow and X those inoculated from the spleen died earlier than those inoculated from the culture. By an oversight the day of the death of the guinea-pig Manila A was not recorded.

Graduated doses of culture.—In the foregoing experiment there was practically no attempt made to give an accurate dose of cultures. In the following series we attempted to secure as high a degree of accuracy in dosage as was possible. Agar cultures 48 hours old were used in each case and the entire series of guinea-pigs used for the six old cultures was inoculated in the same afternoon, July 10, 1908. The procedure was as follows:

Normal salt solution (6.5 gm. per liter) was used in making the dilutions. Great care was exercised to break up the culture and make as perfect a suspension as possible. In making the inoculations the weakest dilution (0.000 001 of a loop) was injected first and the others in succession, using the strongest last. This was done to avoid the necessity of using a separate syringe for each dilution or of sterilizing the syringe after each inoculation. The error of carrying over in the syringe a small amount of the weaker dilution could not be ignored. The syringe was, of course, sterilized whenever the inoculation of a series (one strain) was completed. The dilutions were made of such strength as to make it convenient to use 1 c.c. of the suspension in each case. Each dilution after the first was made by mixing 9 c.c. of the normal salt solution with one c.c. of the next strongest dilution. The object was to make the injection subcutaneously into the abdominal wall, but on account of faulty technique the culture was given intraperitoneally in several instances. When this occurred it is so stated in the table.

There are, of course, very evident sources of error in work of this nature. The loop does not take up exactly the same volume of culture each time; sometimes some of the solution will escape at the site of inoculation; the organism may not be uniformly distributed through the medium. A more important source of error than any of these is the varying resistance of different animals. This is well shown in some of the tables.

For purposes of comparison I used four plague cultures isolated in San Francisco; one from a human case, two from rats, and the last from a squirrel. These four cultures had been isolated directly from the tissues of a human being and the lower animals: i. e., they had never been passed through a laboratory animal. The four last-named cultures were inoculated into animals on different days so that the results are not strictly comparable as they are in the case of the old cultures, but it is not believed that any material source of error existed on that account.

Jedda.—Nothing is known about this culture beyond the fact that it has been at the laboratory for at least eight years. The culture used was the fourth generation on agar. A 48-hour growth was used.

TABLE 4.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
349 gm.	0.01 loop	2	Acute plague	Yes	Intraperitoneal
420 gm.	0.001 "	8	" "	"	
387 gm.	0.0001 "	13	Subacute "	No growth	
432 gm.	0.00001 "	8	Acute "	Yes	
410 gm.	0.000001 "	5	" "	"	Intraperitoneal

Manila.—This culture was isolated in April, 1904, and was therefore four years old at the time these inoculations were made but there is no history as to what was the original source of the culture. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 5.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
312 gm.	.01 loop	10	Acute plague	Yes	
438 gm.	.001 "	9	" "	"	
352 gm.	.0001 "	8	" "	"	
424 gm.	.00001 "	11	Subacute "	"	
333 gm.	.000001 "	12	Acute "	No growth	

New York.—This culture was isolated by Passed Assistant Surgeon John F. Anderson, P. H. and M. H. S., Assistant Director of the Hygienic Laboratory in the early part of 1904 and was therefore four years old when used. The culture was isolated from a guinea-pig inoculated from a case of human plague at the New York quarantine. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 6.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
332 gm.	.01 loop	7	Acute plague	Yes	Intraperitoneal
306 gm.	.001 "	6	" "	"	
277 gm.	.0001 "	7	" "	"	
300 gm.	.00001 "	8	" "	"	
345 gm.	.000001 "	8	" "	"	Intraperitoneal

Glasgow.—No history is obtainable, but the culture is known to be at least four or five years old. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 7.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
312 gm.	.01 loop	6	Acute plague	Yes	
432 gm.	.001 "	6	" "	"	
370 gm.	.0001 "	6	" "	"	
396 gm.	.00001 "	8	" "	"	
320 gm.	.000001 "	14	Subacute "	No growth	

Culture X.—This culture has been carried for about one year on artificial media. Nothing is known of its origin except that it was isolated from the spleen of a guinea-pig. The culture used was the sixth generation on agar. A 48-hour-old growth was used.

TABLE 8.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
280 gm.	.01 loop	5	Acute plague	Yes	
295 gm.	.001 "	7	" "	"	
290 gm.	.0001 "	7	" "	"	
290 gm.	.00001 "	6	" "	"	
328 gm.	.000001 "	7	" "	"	

Reedy Island.—This culture was isolated in May, 1906, by P. A. Surgeon John F. Anderson from a case of human plague on a vessel at the Reedy Island Quarantine Station. The culture was obtained from a guinea-pig inoculated from the case. The culture was the fourth generation on agar. A 48-hour-old growth was used.

TABLE 9.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(subcutaneously)				
325 gm.	1 slant	5	Acute plague	Contaminated	
365 gm.	1 loop	5	" "	"	
314 gm.	.1 "	8	" "	Yes	
416 gm.	.01 "	14	Subacute "	Contaminated	
355 gm.	.001 "	10	" "	No growth	
300 gm.	.0001 "	12	" "	" "	

Human No. 171.—This culture was isolated by routine methods by Acting Assistant Surgeon W. B. Wherry directly from the liver of a case of human plague which died in San Francisco in the early part of November, 1907. The culture used was the second generation on agar and was 48 hours old. The animals were inoculated nine months after the original isolation of the culture.

TABLE 10.

August 3, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(cutaneously)				
263 gm.	1 loop	2	Early plague	Cultures not made	Pneumonia
	(subcutaneously)				
298 gm.	1 loop	2	Early plague	Cultures not made	Pneumonia
253 gm.	.01 "	4	Acute "	Yes	
318 gm.	.001 "	7	" "	"	
354 gm.	.0001 "	5	" "	"	
262 gm.	.00001 "	6	" "	"	
322 gm.	.000001 "	5	" "	"	

The first two animals showed beginning lesions of plague, but a pneumonia, probably due to a streptococcus, was the immediate cause of death.

Squirrel No. 1.—This culture was isolated in pure culture directly from the lung of the first plague-infected squirrel found in California. The culture used was the second generation and was a 72-hour agar growth. The culture was isolated six days before the inoculations were made.

TABLE 11.

August 12, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(cutaneously)				
277 gm.	1 loop	4	Acute plague	Yes	
	(subcutaneously)				
322 gm.	.01 loop	5	Acute plague	Yes	
279 gm.	.001 "	5	" "	"	
331 gm.	.0001 "	8	" "	"	
343 gm.	.00001 "	5	" "	"	Intraperitoneal
326 gm.	.000001 "	7	" "	No growth	

Rat No. 66.—This culture I isolated directly from the liver of natural plague rat No. 66 (new series) in San Francisco. The culture used was the fourth generation on agar and was 48 hours old. The animals were inoculated 41 days after the original isolation of the organism.

TABLE 12.

August 10, 1908.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(cutaneously)				
350 gm.	1 loop	5	Acute plague	Yes	
	(subcutaneously)				
425 gm.	.01 loop	3	Acute plague	Yes	Intraperitoneal
481 gm.	.001 "	7	" "	"	
349 gm.	.0001 "	6	" "	"	
420 gm.	.00001 "	7	" "	"	
465 gm.	.000001 "	16	" "	"	
317 gm.	.0000001 "	8	" "	"	

Owing to an error in making the dilutions one was made of one ten-millionth part of a loopful of the culture. This animal died eight days earlier than did the one receiving ten times as much culture.

Rat No. 82.—This culture was isolated from the liver of plague rat No. 82 (new series) at San Francisco. It was of rather more than ordinary interest as it was isolated apparently at or near the end of the epizootic in this city, the last previous case of rat plague having been detected 85 days prior to this one. This culture as is shown by the

TABLE 13.

Weight of Guinea-Pig	Dose and Mode of Inoculation	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
	(cutaneously)				
326 gm.	1 loop	5	Acute plague	Yes	
	(subcutaneously)				
293 gm.	.01 loop	4	Acute plague	Yes	
320 gm.	.001 "	10	Subacute "	"	
251 gm.	.0001 "	4	Acute "	"	
	.00001 "				
278 gm.	.000001 "	9	Subacute "	Cultures negative, but spleen gave rise to typical acute plague in a guinea-pig from which *B. pestis* was recovered.	

tables is quite virulent for guinea-pigs and white rats. There is, however, some evidence that it is less virulent for wild rats than are the other cultures isolated here. My work has not progressed far enough to enable me to speak definitely on this point. The culture used was a second generation 24 hours old and the inoculations were made 12 days after the original isolation of the culture.

The following compilation from the preceding tables shows the day of death of guinea-pigs inoculated with different doses of the several strains used.

TABLE 14.

	Dose and Mode of Inoculation							
	1 loop cutaneously	0.01 loop subcutaneously	0.001 loop subcutaneously	0.0001 loop subcutaneously	0.00001 loop subcutaneously	0.000001 loop subcutaneously	1 culture subcutaneously	Average
Old Cultures:								
Jedda	7	*a*2	8	13	8	*a*5	3	7.8
Manila	6	10	9	8	11	12	7	9.0
New York	8	*a*7	6	7	8	*a*8	5	6.8
Glasgow	7	6	6	6	8	14	5	7.4
X	8	5	7	7	6	7	4	6.3
*b*Reedy Island	7	14	10	12				10.7
Average	7.2	7.0	7.2	8.2	8.2	11	4.8	7.5
New Cultures:								
Rat. No. 66	5	*a*3	7	6	7	16		8.2
Squirrel No. 1	4	5	5	8	*a*5	7		5.8
Human No. 171		4	7	5	6	5		5.4
Rat No. 82	5	4	10	4		9		6.4
Average	4.6	4.3	7.25	5.75	6.5	9.25		6.4

Note.—(*a*). Intraperitoneal not counted in making averages. (*b*). Reedy Island omitted in calculating averages at foot of columns as this culture was clearly of reduced virulence.

VIRULENCE FOR WHITE RATS.

The next experiment was made with white rats. Full-grown animals were used. In each case the animal was inoculated by the cutaneous method with one loopful of three-day-old agar culture, third generation. The records of Human No. 171, Rat No. 66, Squirrel No. 1, Rat No. 82 are given as controls though they were not inoculated at the same time as were the other eight.

TABLE 15.

Culture	Day of Death	Lesions	*B. Pestis* Recovered	Remarks
Jedda	4	Acute plague	Yes	
Manila	4	" "	Cultures not made	
New York	4	" "	Yes	
Glasgow	5	" "	"	
X	3	" "	"	
Reedy Island		None	No	Killed 13th day
Bombay		"	"	" 8th "
Frisco		"	"	" 8th "
Human 171	6	Acute plague	Cultures not made	
Rat 66	4	" "	Yes	Controls
Squirrel 1	4	" "	"	
Rat 82	3	" "	"	

It is evident that the five old cultures that were constantly virulent for guinea-pigs were also quite virulent for white rats. The four cultures isolated in San Francisco were all fully virulent for the white rats.

SUMMARY.

These experiments demonstrate that of eight cultures of *B. pestis* that have been carried on artificial media for long periods five (5) Jedda, Manila, Glasgow, New York, X, are highly virulent, all of the animals inoculated with these cultures having died. One culture, Reedy Island, is not constantly lethal for animals. Two of these cultures, Frisco and Bombay, are practically avirulent.

The cultures isolated in San Francisco in the recent epidemic, Rat No. 66, Rat No. 82, Human No. 171, and Squirrel No. 1, are highly virulent.

The size of the dose and the mode of administration of virulent cultures (cutaneously or subcutaneously) have no marked influence upon the length of time an animal will live after inoculation. In the last statement an exception must be made in the case of such a colossal dose as a whole agar culture which generally kills earlier than the smaller doses, but in the case of such a large dose it is not improbable that an intoxication, owing to the large mass of bacilli introduced, plays a part.

Varying resistance of different guinea-pigs is of more importance than the dose of culture.

Passing the culture through one guinea-pig has no appreciable influence in raising the virulence.

These statements as to virulence refer only to guinea-pigs and white rats. Work is now in progress to determine the virulence of these cultures for wild rats (*Mus norvegicus*).